PIEZOELECTRIC MICROCANTILEVER SENSORS FOR BIOSENSING

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Prior Publication Data
US 2015/0105294 A1 Apr. 16, 2015

Related U.S. Application Data
Division of application No. 12/514,941, filed as application No. PCT/US2007/085771 on Nov. 28, 2007, now Pat. No. 8,927,259.

Provisional application No. 60/867,538, filed on Nov. 28, 2006.

Int. Cl.
C12M 1/34 (2006.01)
C12M 3/00 (2006.01)

(Continued)

ABSTRACT

A piezoelectric microcantilever for sensing compounds or molecules. The piezoelectric microcantilever, may include at least one electrode, an insulation layer, a receptor, an immobilization layer, a non-piezoelectric layer and a piezoelectric layer. The sensor is capable of self-actuation and detection. The piezoelectric layer may be constructed from a highly piezoelectric thin lead magnesium niobate-lead titanate film, a highly piezoelectric thin zirconate titanate film, a highly piezoelectric lead-free film. Methods of using the sensors and flow cells and arrays including the sensors are also described.

20 Claims, 26 Drawing Sheets
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(a) Grow SiO$_2$ layer by thermal oxidation on Si wafer

(b) Deposit TiO$_x$/Pt bottom electrode by reactive sputtering

(c) Deposit PZT thin film on Si/SiO$_2$/TiO$_x$/Pt substrate

(d) Deposit top electrode and Ni layer on PZT

(e) Etch and pattern PZT by chlorine based ICP

(f) Etch and pattern TiO$_x$/Pt by chlorine based ICP

(g) Deposit and pattern Au bonding pad on electrode

(h) Etch Si from back side by KOH

(i) Etch SiO$_2$ from the front side by RIE to release cantilever

Figure 3
Commercial PZT based
PMN-PT based
PZT thin film based

Figure 4(b)
Figure 6
Figure 9
Figure 10

- Δf (kHz)

Total Number of Spores Present

PEMS-A

PEMS-B
FIG. 11A

FIG. 11B

TIME (MIN)

FREQUENCY SHIFT (Hz)

- 2.19 μg/ml
- 4.38 μg/ml
- 8.76 μg/ml
- 17.5 μg/ml
- 26.1 μg/ml
Figure 12
Figure 13(a)

Figure 13(b)
Figure 13(c) - $\Delta f$ (Hz) vs. Time (Hz)

- Anti-GP120
- Anti-Her2
- Anti-PSA

Figure 13(c) - Phase Angle (degrees) vs. Frequency (kHz)

Figure 14(a)
FIG. 15A
Figure 15(b)
Figure 17(a)

Figure 17(b)
Figure 17(c)

Figure 18
FIG. 19
Figure 21(a)
Figure 21(b)
**FIG. 22A**

**FIG. 22B**
PIEZOELECTRIC MICROCANTILEVER SENSORS FOR BIOSensing


STATEMENT OF GOVERNMENT INTEREST

This invention was reduced to practice with Government support under Grant No. R01 EB000720 awarded by the National Institutes of Health; the Government is therefore entitled to certain rights to this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to piezoelectric microcantilever sensors for biosensing. More specifically, the invention relates to highly sensitive piezoelectric microcantilevers capable of determining the presence and/or mass of organic compounds. Applicable fields of use may include biodetection, food safety and pathogen detection.

2. Description of the Related Technology

Current biosensing technologies utilize quartz crystal microbalances (QCM), silicon microcantilevers, electrochemical enzyme immunoassays, fluorescence, laser-based or fiber-optics-based methods, amplification schemes such as polymerase chain reaction (PCR), or bound metal particles to determine the presence and/or mass of organic compounds. These techniques, however, fail to provide quantitative, efficient or highly sensitive detection. In addition to lacking sensitivity, they are also incapable, in some cases, of simultaneously monitoring multiple compounds or being used in high throughput array applications.

TABLE 1

<table>
<thead>
<tr>
<th>Detector Type</th>
<th>Detection Sensitivity</th>
<th>Label-free</th>
<th>Direct</th>
<th>In-situ</th>
<th>Rapid</th>
<th>High-throughput</th>
<th>Multiplexing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEMS</td>
<td>$10^{-12} - 10^{-18}$</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>QCM</td>
<td>$10^{-6}$</td>
<td>yes</td>
<td>yes</td>
<td>Yes</td>
<td>No</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Silicon microcantilever</td>
<td>$10^{-12}$</td>
<td>yes</td>
<td>no</td>
<td>No</td>
<td>No</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Optical fiber fluorescence</td>
<td>$10^{-10}$</td>
<td>no</td>
<td>no</td>
<td>No</td>
<td>Yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>ELISA²</td>
<td>$10^{-12}$</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>Yes</td>
<td>No</td>
<td>no</td>
</tr>
<tr>
<td>SPR³</td>
<td>$10^{-12}$</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

Of these technologies, QCM, which utilizes thickness-mode resonance sensing, is one of the most common commercially available biosensing technologies. Detection sensitivity of a QCM is related to the resonance frequency and the thickness of the quartz membrane. A resonance frequency of about 5 MHz, corresponding to a quartz membrane thickness of 330 µm, enables a minimum detectable mass density of about $10^{-12}$ g/cm². Sensitivity is therefore generally limited to a range of about $10^{5}$ g/Hz.

To increase sensitivity, some biosensors utilize silicon-based microcantilevers, which offer a sensitivity of approximately $10^{-12}$ g/Hz, about three orders of magnitude higher than QCMs. Advantageously, silicon microcantilevers are also widely available and may be easily integrated with existing silicon fabrication methodologies. Most silicon microcantilevers, however, rely on complex external optical components for deflection detection, an external driving mechanism for actuation and also require laser alignment. Moreover, because they are not piezoelectric, silicon microcantilevers are inferior for in-solution sensing, yielding low resonance peaks upon immersion in a solution.

Piezoelectric cantilevers, in comparison, use electrical means for detection and are not encumbered by the complexity and mass of the silicon-based sensors. Constructed from lead zirconate titanate (PZT), they are capable of electrical self-excitation and self-sensing for in-situ electrical detection. Currently, piezoelectric biosensors are millimeter-size cantilevers made by bonding commercial PZT to non-piezoelectric substrates such as stainless steel, titanium or glass. Although capable of in-situ biosensing, these millimeter-size cantilevers lack the desired sensitivity for such applications.

Thin-film-based PZT microcantilevers, such as those disclosed in JP-07027559 A2 and U.S. Pat. No. 7,084,554, are highly sensitive instruments. U.S. Pat. No. 7,084,554, in particular, discloses a thin piezoelectric film biomorph capable of being formed as a cantilever. The biomorph may be composed of a piezoelectric thin PZT film of about 1-10 µm in thickness for the purpose of increasing the working frequency range of micro-electro-mechanical dimensioned (MEMS) systems. The patent further teaches that the piezoelectric thin film may be fabricated by thin film fabrication methods such as a sol-gel method, sputtering, hydrothermal methods, chemical vapor deposition (CVD) or another thin film fabrication method, followed by low temperature annealing and dry etching, plasma etching or patterning by wet chemical etching (See col. 5, lines 55-64 of U.S. Pat. No. 7,084,554).

These piezoelectric microcantilevers, however, are incapable of in-situ electrical detection due to degradation of resonance peaks in solution.

Recent advances in thin-film PZT microcantilevers incorporate an electrical insulation layer that prevents liquid damp-
In a first aspect, the present invention is directed to piezoelectric microcantilever sensors useful for detecting organic compounds. The piezoelectric microcantilever includes a piezoelectric layer, a non-piezoelectric layer and a receptor. The microcantilever may be constructed from piezoelectric lead magnesium niobate-lead titanate \((\text{Pb(Mg}_{0.33}\text{Nb}_{0.67})\text{O}_{3})\), \((\text{PbTiO}_{3})\), \((\text{Pz})\), or \((\text{PMN-PT})\) which typically has a thickness not greater than 70 \(\mu\)m with a dielectric constant of at least 1600.

In a second embodiment, the piezoelectric layer of the microcantilever is constructed from lead zirconate titanate \((\text{PZT})\), which typically has a thickness not greater than 4 \(\mu\)m with a dielectric constant of at least 1600.

In another aspect, the present invention is directed to methods for bioterrorism defense, food safety and pathogen detection.

**SUMMARY OF THE INVENTION**

As bioterrorism threats become more prevalent, there is a growing need for reliable in-situ detectors capable of efficiently detecting multiple biological agents in real time.

Advancements in biosensing accuracy, sensitivity, multi-component detection are also potentially useful in the health sciences for early detection and prevention of diseases. Breast cancer, for example, is the second leading cause of death for women. Although a number of potential breast cancer markers, such as HER2 (HER2/neu, c-erb-B-2), EGFR, CA-15-3, CA27.29, uridine diphospho-glucuronosyl transferase (uPAR), carcinoembryonic antigen (CEA), α-fetoprotein (AFP), and cytokeratins, are known, no blood test or method for detecting these markers currently exists. Mammography is frequently inadequate and often produces false positives leading to unnecessary biopsies. Therefore early detection methods for various cancers, for conditions lacking adequate diagnostic means, like breast cancer, that are capable of accurately, effectively and non-invasively identifying and quantifying pathogens and other disease markers are also needed.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 is a cross section of one embodiment of a piezoelectric microcantilever in accordance with the present invention.

FIG. 2(a) is a graph of piezoelectric coefficient, \(-d_{11}\), versus electric field for PMN microcantilevers in accordance with the present invention.

FIG. 2(b) is the resonance spectrum of a PMN—PT PEMS.

FIG. 3 is a schematic showing a preferred micro-fabrication process for PMN—PT/SiO\(_2\) microcantilevers in accordance with the present invention.

FIG. 4(a) is a graph of quality value (Q) versus cantilever length for PZT cantilevers in accordance with the present invention.

FIG. 4(b) is a graph of quality value (Q) versus cantilever length for PZT and PMN—PT PEMS in accordance with the present invention.

FIG. 5(a) depicts a flow cell system which can be used in conjunction with the cantilevers of the present invention.

FIG. 5(b) depicts a 3.5 in. by 7.5 in. portable PEMS sensor capable of working with 8 sensors and powered by a 9-V battery.

FIG. 6 is a graph of \(\Delta m/\Delta f\) as a function of cantilever length.

FIG. 7 is a graph of resonance frequency shift as a function of time for the microcantilever PEMS-B.

FIG. 8 is a graph of resonance frequency shift as a function of time for the microcantilever PEMS-B using a resonance peak of 285 kHz.

FIG. 9 is a graph of resonance frequency shift as a function of time for the microcantilever PEMS-A.

FIG. 10 is a graph of resonance frequency shift as a function of the total number of BA spores for both microcantilevers PEMS-A and PEMS-B.

FIG. 11(a) depicts an experimental setup for determining the resonance frequency shift for a 1 mm long PZT/glass cantilever having a 2 mm long glass tip.

FIG. 11(b) is a graph of resonance frequency shift as a function of time of the PEMS of FIG. 11(a).

FIG. 12 is a graph of frequency shift as a function of PSA concentration for various cantilevers.

FIG. 13(a) is a graph of resonance frequency shifts of three piezoelectric cantilevers as a function of time in a HER2 solution.

FIG. 13(b) is a graph of resonance frequency shifts of three piezoelectric cantilevers as a function of time in a PSA solution.

FIG. 13(c) is a graph of resonance frequency shifts of three piezoelectric cantilevers as a function of time in a gp120 solution.

FIG. 14(a) shows the resonance spectra of a parylene insolated PEMS placed in static and 1 ml/min flow solutions of PBS.

FIG. 14(b) shows graphs of peak shift as a function of time for a parylene insolated PEMS placed in static and 1 ml/min flow solutions of PBS.

FIGS. 15(a)-15(b) are resonance spectra of a MTMS insulated PZT PEMS placed in a 0.5 ml/min flow solution of PBS.

FIG. 16(a) shows a schematic diagram of binding an NHS ester of the SMCC to a primary amine of the scFv.

FIG. 16(b) shows the formation of a thioether bond between the maleimide of the SMCC and the sulfhydryl group of the PES on the sensor surface.

FIG. 17(a) shows a graph of resonance frequency as a function of time during scFv immobilization, PBS rinsing, blocking in a 10 mg/ml of BSA solution, and detection in a 100 ng/ml pure HER2 solution for a PMN—PT/Sn PEMS insulated by MPS.

FIG. 17(b) shows resonance spectra at various time frames of FIG. 17(a).

FIG. 17(c) shows a graph of resonance frequency as a function of time during scFv immobilization, PBS rinsing, blocking in a 10 mg/ml of BSA solution, and detection in a 100 ng/ml of HER2 solution and 1 mg/ml BSA solution for a PMN—PT/Sn PEMS insulated by MPS.

FIG. 18 is a graph of resonance frequency shift as a function of time of a PMN—PT/Sn PEMS insulated with MPS with scFv immobilized via SMCC coupling at 5, 10, and 50 ng/ml of HER2. The inset is a blow-up that shows the resonance frequency shift at 5 and 1 ng/ml HER2.

FIG. 19 is a graph of resonance frequency as a function of time of a one-sided PMN—PT/in PEMS during the various stages of detection of HER2 at 1 ng/ml in a background of 1 mg/ml BSA.

FIG. 20 is a graph of resonance frequency as a function of time showing the detection of HER2 in solutions containing diluted (1:40) fetal bovine serum and HER2 in a concentration of about 110 pg/ml, 30 pg/ml, 30 pg/ml or 10 pg/ml using a PMN—PT/Cu PEMS.

FIG. 21(a) is a graph of resonance frequency shift as a function of time showing the detection of HER2 in a solution of diluted (1:40) fetal bovine serum containing a 6 ng/ml concentration of HER2 using three different receptors.
FIG. 21(b) is a graph of resonance frequency shift as a function of time showing the detection of HER2 in a solution of diluted (1:40) fetal bovine serum containing a 60 ng/ml concentration of HER2 using three different receptors.

FIG. 21(c) is a graph of resonance frequency shift as a function of time showing the detection of HER2 in a solution of diluted (1:40) fetal bovine serum containing a 600 ng/ml concentration of HER2 using three different receptors.

FIG. 22(a) is an optical micrograph of the PZT/glass PEMS array used in the CP detection and

FIG. 22(b) is a graph of resonance frequency shift as a function of time showing the detection of CP at a concentration of about 1 CP/ml using 4 different PEMS.

FIG. 23(a) is an SEM micrograph a 40 μm long PZT/SiO2 PEMS.

FIG. 23(b) is a graph of resonance frequency shift versus relative humidity of a 40 μm long PZT/SiO2 PEMS.

DETAILED DESCRIPTION OF THE PREFERRED EMDOIMENTS

Piezoelectric microcantilever sensors (PEMS) are mass sensors that use an electrical means for detection. Receptors are coated on the surface of PEMS to bind molecules of interest. The PEMS detects a change in mass because newly bound target molecules shift the mechanical resonance frequency of the device. By monitoring the resonance frequency shifts, a PEMS is capable of rapid, label-free, in situ quantitative detection of organic compounds or molecules including pathogens, antigens and proteins in a small volume solution (e.g. 100 μl) or in a cell culture using simple all-electrical measurements. PEMS are capable of electrical actuation and detection and may also be constructed as an array for simultaneous monitoring of multiple target compounds or molecules.

FIG. 1 shows the basic structure of a microcantilever sensor. A PEMS includes a conductive element 1 and a second conductive element 2 (bottom electrode), electrically insulating layer 3, receptor immobilization layer 4, receptors 5, at least one non-piezoelectric layer 6, and at least one piezoelectric layer 7. The PEMS shown in FIG. 1 also includes electrical leads 9.

Conductive elements 1, 2 may be any element capable of conducting an electrical signal from the piezoelectric layer to a device for detecting that signal. In a preferred embodiment, conductive elements 1 and 2 are electrodes which may be constructed from any conductive material. Preferably, the first electrode 1 is constructed from Au/Cr or Pt/Ti and subsequently patterned in several regions. The second electrode 2 is preferably constructed from Pt/TiO2 on SiO2 for PZT/SiO2 PEMS or Pt/Ti on a metal substrate or non-piezoelectric layer and subsequently patterned as well.

In order to maintain functionality in solution by preventing conduction, it may be useful to electrically separate or buffer conductive element 1 and second conductive element 2. Conductive element 1 is patterned slightly smaller than the piezoelectric layer 7 to ensure complete insulation of the edges and corners thereof. Any electrically insulating layer 3 may be used as a coating to achieve electrical separation or buffering.

In one embodiment, insulating layer 3 may comprise a 1.5 μm thick parylene (poly-para-xylylene) coating deposited on an electrode by chemical vapor deposition. When placed in static and 1 ml/min flow rate of PBS solution, a parylene insulating layer essentially prevents background resonance frequency shifts greater than 30 Hz and 60 Hz, respectively, over a period of 30 minutes. As a result, insulating layer 3 enables complete submersion of the microcantilever for in situ or in-solution detection while maintaining a Q value (quality value) greater than 35. For the purposes of this patent application, Q value is defined as the ratio of the resonance frequency to the resonance peak width at half the peak height.

Alternatively, a PEMS may be insulated using self-assembled monolayers with hydrophobic properties, preferably methytrimethoxysilane (MTMS) or a combination of MTMS with parylene coatings of varying thicknesses, may also be used. When immersed in a PBS solution, an MTMS insulated piezoelectric microcantilever yields strong resonance peak intensities and prevents background resonance frequency shifts greater than 30 Hz over a period of 30 minutes.

Other insulation materials may include 3-mercaptopropyl trimethoxysilane, Al2O3, SiO2, and any functional hydrophobic silane, having a hydrophobic group selected from the group consisting of allyl, phenyl, alky1 halide, alkene, alkane, and sulfhydryl. In a preferred embodiment, the insulation material is mercaptopropylsilane (MPTS), which can also function to immobilize a receptor on the cantilever. The insulating materials may also include any combination of any of MTMS, MPTS, parylene, 3-mercaptopropyl trimethoxysilane, Al2O3, SiO2, any functional hydrophobic silane having a hydrophobic group selected from the group consisting of allyl, phenyl, alkyl halide, alkene, alkane, and sulfhydryl, or a combination thereof.

Receptors 5 may be densely packed and immobilized onto, for example, a bi-functional linker modified sensor surface. Any receptor, such as specially synthesized cavitrans, DNA oligonucleotides, proteins, single chain variable fragments (scFv), enzymes, and antibodies to cells, antigens, pathogens, viruses, parasites, or combinations thereof may be bound to the sensor surface. For example, when trying to detect tumors, monoclonal and dimeric anti-tumor scFv molecules, which are composed of variable light and heavy chains of antibody molecule anti-ECV scFv, that react to cancer markers may be bound to the electrodes. Similarly, when trying to detect Bacillus anthracis ("BA"), antibodies specific to BA spore surface antigens may be immobilized on the electrodes.

Any means of adhering receptors 5 to the sensor surface may be utilized. In a preferred embodiment, receptors 5 are bound to the electrodes using an immobilization coating 4, such as self assembled monolayers ("SAM"), MPTS and bi-functional linkers. In one embodiment, for purposes of binding scFv, the immobilization coating may be a self assembled monolayer of 3-mercaptopropionic acid (MPA) on a copper and gold-coated electrode activated with 1-ethyl-3-(3-dimethylaminopropyl)carboimide hydrochloride (EDC) and 1 mg/ml N-hydroxysuccinimide (NHS).

The PEMS also includes at least one non-piezoelectric layer 6, which may be fabricated from any compatible material, including a ceramic material, a polymeric material, a metallic material or combinations thereof. Preferably the non-piezoelectric layer 6 is fabricated from silicon dioxide (SiO2) and silicon nitride (Si3N4) for PZT-thin film based PEMS. For example, a silicon nitride coating on single crystal silicon wafer may be prepared by low pressure chemical vapor deposition. A low stress silicon dioxide layer may subsequently be deposited on the silicon nitride layer by growing silicon dioxide films using low temperature oxide deposition or plasma enhanced chemical vapor deposition. For PMN—PT-based PEMS, the non-piezoelectric layer can be any ceramic, metallic, or polymeric layer. A metallic layer such as Cu, tin, Ni, Ti, etc., or any combination is preferred because it can be provided by simple electroplating.
Non-piezoelectric layer 6 may be bonded to a shorter piezoelectric layer 7 so that the portion of non-piezoelectric layer 6 extending beyond the end of piezoelectric layer 7 forms a non-piezoelectric tip. Both piezoelectric layer 7 and non-piezoelectric layer 6 may be attached to a clamp. In an alternative embodiment, piezoelectric layer 7 may extend beyond non-piezoelectric layer 6, forming a piezoelectric tip. Optionally, the PEMS may be constructed so that neither piezoelectric layer 7 nor the non-piezoelectric layer 6 extends beyond the other. In order to achieve the best results, one of the piezoelectric 7 and non-piezoelectric layers 6 preferably extends beyond the other to form a tip. A PEMS may also include multiple piezoelectric and non-piezoelectric layers. For example, a non-piezoelectric layer may be placed between two piezoelectric layers or a piezoelectric layer may be placed between two non-piezoelectric layers.

A significant aspect of the microcantilever device is the fabrication of a highly piezoelectric layer 7, which enables electrical detection and actuation within the cantilever. The piezoelectric layer may function as a driving element, vibrating element and sensing element. Applying an AC voltage (input) across the piezoelectric layer bends and vibrates the PEMS, which in turn induces a piezoelectric voltage that produces readily detectable changes in the magnitude and phase of the output voltage. The resonance frequency of the PEMS is obtained by monitoring the maximum of the phase shift of the output voltage. This measurement is accomplished all-electrically, i.e., electrical actuation and electrical sensing.

Piezoelectric layer 7 may be constructed from any piezoelectric material, including a lead-free piezoelectric material such as (Na0.5K0.5)0.85Li0.15Nb0.95O3, (BaNb2O6)0.95, and (Pb(0.5x)Sr(0.5x)0.95Nb0.05O3)0.95 [hereinafter “SB-NKNL”]. Such lead-free piezoelectric layers may be fabricated by well-known processes. The resulting Suspensions have previously been used to formulate PMN—PT freestanding films of about 8-75 µm thick upon sintering at 1000°C. Preferably, the thickness is about 1 µm-127 µm, more preferably less than about 50 µm, and most preferably less than about 8 µm. The precursor-suspension method may also be used to produce freestanding PMN—PT films having a thickness of less than 8 µm or larger than 75 µm. Typically, the freestanding PMN—PT films have ferroelectric sensitivity of at least 2×10⁻¹⁴ C/N, dielectric constants greater than 1000, saturated polarization of about 30 µC/cm², remnant polarization of 25 µC/cm² and a Q value as high as 300 and as low as 20. Therefore PMN—PT microcantilevers are capable of generating higher-mode resonance peaks resulting in enhanced sensitivity detection.

Further, PMN—PT freestanding films with thicknesses of 8 µm or 22 µm were found to exhibit an extremely high electric-field enhanced piezoelectric – d33, coefficient of 2000 nm/V at E=10 kV/cm, as shown in FIG. 2(a), which is about 7 times that of the bulk PMN—PT and commercial PZT, and which also exceeds the piezoelectric coefficient of specially-cut single-crystalline PMN—PT. The direction of the triangles in FIG. 2(a) indicate whether the results were obtained when the field was ramped up or down. The piezoelectric coefficient – d33 is calculated according to (Equation 1),

\[ d_{33} = \frac{b_{23} \cdot E_{1} \cdot E_{2}}{V_{0} \cdot L \cdot E_{i} \cdot E_{f}(t_{1} + t_{2})(1 - v)} \]  

(1)
using a sol-gel process. FIG. 3 depicts a preferred microfabrication procedure for constructing PZT/SiO₂ piezoelectric microcantilever sensors.

One advantage of this invention is the ability to construct a microcantilever from one piece of PZT/SiO₂ film without having to separately attach the non-piezoelectric 6 and piezoelectric layers 7. Because the cantilevers thus fabricated have clean interfaces between the PZT/SiO₂ layer and the electrodes, the cantilevers exhibit high Q values. The resultant clean geometry of the microcantilever increases sensitivity and facilitates the manufacturing process.

The self-actuating and self-detecting PEMS of the present invention overcomes a significant problem of the prior art, miniaturizing microcantilevers without losing high resonance-peaks. In the present invention, the novel PMN and PZT micro-fabrication methods produce thin piezoelectric PEMS having enhanced sensitivity, as evidenced by the large dielectric constants, piezoelectric coefficients and Q values.

Q values are an excellent indicator of cantilever sensitivity. Although it is expected that the Q value of silicon microcantilevers decreases with cantilever size, FIG. 4 shows that the Q value of a commercial-PZT-based piezoelectric microcantilever remains approximately the same even though the microcantilever length varies over two orders of magnitude due to the polycrystalline composition of the piezoelectric layer 7. The piezoelectric layer 7 of the present invention therefore utilizes polycrystalline compositions such as PMN—PT and PZT in order to miniaturize PEMS without losing sensitivity. For example, by reducing PZT PEMS to a length-scale of 300 μm and 50 PMN—PT and PZT PEMS are capable of retaining detection sensitivity greater than 10⁻¹⁵ and 10⁻¹⁶ g/Hz, respectively. Freestanding PMN—PT PEMS 500-700 μm in length are capable of achieving Q values higher than 300. Additionally, PEMS with a thickness of less than 5 μm and a length of less than 120 μm long, may achieve a sensitivity better than 10⁻¹⁶ g/Hz.

To further increase sensitivity and expedite the detection process, the PEMS may be immersed in a flowing solution for in-solution detection. The PEMS is preferably situated in a flow cell system to enable tailored, rapid and simultaneous detection and quantification of multiple organic compounds or molecules.

FIG. 5(a) shows a flow cell system 10, with a PEMS holder/measuring unit 11, having a total volume of less than 0.03 ml, pump 12, and a mechanism for controlling temperature and humidity (not shown). The flow cell 10 may attain flow rates of up to 1 ml/min. The total volume of the flow cell, number of channels and flow rate may vary depending upon the number of compounds to be measured. The flow cell 10 may cooperate with a portable PEMS unit, shown in FIG. 5(b), which has multiple channels for the simultaneous quantification of multiple receptor specific molecules.

Another means for further enhancing sensitivity is by increasing humidity. The mass change per unit area per percent humidity change of PZT PEMS is estimated to be about 1.2×10⁻¹⁴ g/Hz/mm²/% humidity. The sensitivity of PMN PEMS by comparison is known to be about three times greater than that of PZT PEMS.

The resultant PEMS are chemically inert, thermally stable and miniaturized to enhance sensitivity. They function by binding target molecules that react to the receptors immobilized on the electrodes. The corresponding change in mass shifts the mechanical resonance frequency of the microcantilever. The PEMS is capable of detecting these shifts in resonance frequency by monitoring the 1⁻mode flexural

resonance frequency f., which is related to the effective spring constant, K, and effective mass, M., of the piezoelectric cantilever at the tip as shown in Equation 2.

\[ f = \frac{1}{2\pi} \sqrt{K/M} \]

The binding of a receptor specific molecule to the cantilever surface changes the cantilever mass and the cantilever spring constant. The resonance frequency shift Δf, expressed in Equation 3,

\[ \Delta f = f - f = \frac{\Delta m}{2M} + \frac{\Delta k}{2K} \]

where Δm and Δk denote the mass change and the effective spring constant, model the functionality of the microcantilever. FIG. 6 shows the Δm/Δf of PMN—PT/Cu PEMS constructed from 5 μm and 22 μm thick PMN—PT films. The full circles and full squares of FIG. 6 represent results obtained with millimeter long PEMS, with and without a stainless steel tip, respectively. The Δm/Δf of these PEMS deviates from the solid line by more than three orders of magnitude, thereby indicating that reducing the PEMS thickness and dimension binding-related stress greatly enhances the resonance frequency shift.

These PEMS may be used for various sensing applications such as solid-liquid transition detectors, liquid viscosity and density sensors, mass sensors for in situ and in-water detection. PEMS may generally be used for detection of any molecule or organic compound.

The PEMS technology may be particularly promising for the detection of bioterrorism agents. Antibody receptors specific to at least one bioterrorism agent may be bound to an electrode and used to detect the presence of a bioterrorism antigen. In addition to identifying the existence of a bioterrorism agent, it may also be used to quantify the concentration of the agent.

Additionally, PEMS may be useful in the health sciences as a diagnostic instrument. It may be used as a means for early detection of cancers and other diseases. It may also be used to monitor the progress of the disease throughout treatment. The PEMS may be incorporated in a portable device and used as a noninvasive means for testing blood and other bodily fluids for various pathogens, infectious agents and other markers indicative of disease.

PEMS may also be particularly applicable for the food science and food manufacturing industry. PEMS may be used as a diagnostic instrument for detecting pathogens or other disease agents present in food supplies and prepared or processed foods. Additionally, it may also be useful in manufacturing plants and food service industries as a means of intermittently checking food products during different phases of food preparations thereby preventing contamination and the spread of bacterial or viral diseases such as salmonella and E. coli.

EXAMPLES

Example 1

PMN—PT/Sn PEMS were used in situ to detect the presence of Bacillus anthracis (BA), a bioterrorism agent. Two PEMS, PEMS-A (500 μm long, 800 μm wide, with a 22 μm
thick PMN—PT layer, a 20 μm thick tin layer and a $1 \times 10^{-12}$ g/Hz mass detection sensitivity) and PEMS-B (350 μm long, 800 μm wide, with an 8 μm thick PMN—PT layer, a 6 μm thick tin layer and a $3 \times 10^{-13}$ g/Hz mass detection sensitivity) were inserted in a flow cell through which a solution containing BA was pumped.

The experiment demonstrates that in phosphate buffered saline (PBS) solution, the PEMS were capable of achieving Q values ranging from 50 to 75. PEMS-A exhibited resonance frequency shifts of 2100±200, 1100±100 and 700±100 Hz at concentrations of 20000, 2000, and 2000 spores/ml or 16000, 1600, and 160 total spores, respectively. PEMS-B exhibited resonance frequency shifts of 2400±200, 1500±200, 500±150 and 200±100 Hz at concentrations of 20000, 2000, 100, and 45 spores/ml or 16000, 1600, 80, and 36 total spores, respectively.

The PEMS were fabricated from PMN—PT freestanding films 22 μm and 8 μm in thickness and electrically insulated with methyltrimethoxysilane (MTMS) coatings on the tin surface. A 30 nm thick nickel layer with a 15-50 nm thick chromium bonding layer was first deposited on the opposite face of the PMN—PT freestanding film by evaporation using an E-gun evaporator as the electrode. A thin non-piezoelectric layer was then electroplated on the nickel surface at a rate of 0.5 μm/minute, using tin sulfate in a plating solution. A 150 nm thick platinum on a 10 nm thick titanium layer was evaporated on the other face of the film as the other electrode. The resultant PMN—PT/Sn bilayer was embedded in wax and cut into the cantilever shape with a wire saw. Gold wires were attached to the top and bottom electrodes using conductive glue for electrical connection. The PMN—PT/Sn strips were finally glued to a glass slide or a plastic substrate with epoxy to form the microcantilever geometry.

To insulate the tin electrode of these PMN—PT/Sn PEMS, the PEMS were first soaked in a diluted (1:40 in water) piranha solution (one part of 98% sulfuric acid with one part of 30% hydrogen peroxide) at 20° C. for 2 mN. Methyltrimethoxysilane (MTMS) was then spin coated on the tin surface at 2000 rpm and cross linked (pH=9.5 for 2 hr) twice to form two MTMS coatings on the tin surface.

For antibody immobilization, the platinum electrode was cleaned with a 1:40 diluted piranha solution for 2 min followed by soaking the PEMS in a 2 mM 3-mercaptopyropionic acid (MPA) for 2 hr to form a MPA monolayer on the Pt surface. The carboxyl group of the MPA was then activated by a solution of 2 mM N-Ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM N-hydroxysuccinimide (NHS) in water to allow covalent bonding of primary amines on the anti-BA-spore antibody to the MPA on the platinum surface.

Anti-BA IgG antibodies were then immobilized on the platinum electrode of the PMN—PT layer by first mounting the immersed PEMS with activated MPA in a specifically designed holder and vertically inserting the PEMS into the center of a custom polycarbonate flow cell, 1 mm wide by 1 mm high by 10 mm long with its major faces parallel to the flow and capable of holding a liquid volume of 0.8 ml. A baseline of pure phosphate buffered saline (PBS) solution was run through the flow cell for several minutes before injecting a 600 nM antibody solution into the flow cell for 40 min, after which the flow system was once again rinsed with pure PBS. The flow rate used for antibody immobilization and subsequent BA binding was 1 ml/min, which corresponded to a Reynolds number of 1.6 and a flow speed of 2.5 cm/second over the PEMS face.

Subsequently, 0.8 ml of the desired concentration of UV-killed Sterne strain 7702 BA spores were injected into the flow system and run for 30 minutes. BA spores were then released from the sensor surface in a mixture of glycine and HCl at pH of 2.5 for 7 min. The total volume of the liquid utilized was only 0.8 ml; thus, the actual number of spores available for detection by the cantilever is actually only 80% of the indicated concentration. Scanning electron microscopy (SEM) was performed to confirm spore detection and sensitivity calculations.

FIG. 7 shows the time evolution of a 404 kHz resonance frequency of PEMS-B during antibody immobilization, BA spore detection, and spore release. The antibody solution was introduced at t=0. The resonance frequency shift during t=0-15 mM corresponds to the immobilization of the antibody to the PEMS platform surface. The resonance frequency decreased 0.4 kHz from 403.8 kHz at t=0 min to 403.4 kHz at t=15 min due to the binding of the antibody to the MPA on the platinum surface. This resonance frequency shift saturated at t=10 min and Δf=−0.4 kHz, indicating the immobilization took about 10 min, approximately the same as dipping in static solution. At t=15-20 min the antibody solution was replaced with PBS. As can be seen, there was no significant shift in that period. At t=20 min, when 0.8 ml of a 20,000 spores/ml (16,000 total spores) BA suspension was introduced into the flow cell, the resonance frequency rapidly decreased from 403.4 kHz at t=20 min to 401 kHz at t=40 min with a Δf=−2.4 kHz. This resonance frequency shift during t=20-40 min corresponded to the binding of spores to the antibody immobilized on the PEMS surface. At t=60-65 min, the spore suspension was replaced by PBS, again resulting in no significant resonance frequency shift in that period. From t=65-72 min, the release of the bound spores from the sensor surface in glycine/HCl solution was performed resulting in a recovery (up-shift) to 403.1 kHz at t=72 min, nearly the value before the detection. The second detection period with 0.8 ml of a suspension of 20,000 spores/ml (16,000 total spores) was started at t=72 min. As can be seen, the second detection at the same concentration exhibited a resonance frequency shift similar to the first detection. FIG. 7 therefore shows that the PEMS may be completely submerged in PBS while detecting the binding of proteins and spores to the sensor surface by monitoring the resonance frequency down-shift of a particular peak. Conversely, the release of the antigen can be detected by monitoring the resonance frequency up-shift as is shown during the release of the spores by glycine/HCl solution.

FIG. 8 shows the plot of a resonance frequency shift of PEMS-B versus time using a resonance peak of 285 kHz at BA spore concentrations of 20,000 spores/ml (16,000 total spores), 2000 spores/ml (1600 total spores), 100 spores/ml (80 total spores), 45 spores/ml (36 total spores) and 12 spores/ml (10 total spores). Also shown inset in FIG. 8 are several in-PBS resonance spectra of PEMS-B during the BA spore detection at 20,000 spores/ml concentration. The solid line, the dashed line, and the dashed-dotted line represent the resonance spectrum at t=0, 15 and 30 min, with Q values of 75, 65 and 69 respectively (where Q is ratio of the resonance frequency to the resonance peak width at half of the peak height). As can be seen in FIG. 8, resonant frequency shifts of 2400±200, 1500±200, 500±100, and 200±100 Hz were observed at t=30 min for 16000, 1600, 80, and 36 total spores, respectively. As the concentration was reduced to 12 spores/ml (10 total spores), no discernable resonance frequency shift could be resolved from the sensor noise.

FIG. 9 shows a plot of the resonance frequency shift of PEMS-A versus time for concentrations of 20,000 spores/ml (16,000 total spores), 2000 spores/ml (1600 total spores) and 200 spores/ml (160 total spores). The insert in FIG. 9 shows the in-PBS resonance spectra of PEMS-A at t=0, 15, and 30 min with Q=62, 50, 55 as the solid, dashed, and
dashed-dotted lines, respectively during the detection of BA spores at 16000 total spores. As is shown in FIG. 9, at t=30 min these concentrations yielded frequency shifts of 2100±200, 1100±100, and 700±100 Hz, respectively. FIG. 4 also reveals that, for the lower spore levels of 160 and 1,600, the resonance frequency shift begins to level off at approximately 20 minutes while for the higher spore level of 16,000, the resonance frequency continues to decrease during the entire detection period as similar to the situations with PEMS-B. The resonance frequency shifts versus total number of BA spores of both PEMS-A and PEMS-B are summarized in FIG. 10. The experimentally obtained mass detection sensitivities are more than 100 times more sensitive than the theoretical mass sensitivity values suggested by considering simply the mass loading effect alone.

**Example 2**

A PZT/SiO₂ piezoelectric cantilever sensor (PECS) 0.5 mm long and 2 mm wide, 5 MHz QCM and various PMN—PT PEMs were tested for in-situ detection of prostate specific antigen (PSA). The tip of each cantilever was partially immersed in PSA solutions of 26.1, 17.5, 8.76, 4.38, and 2.19 µg/ml to a depth of approximately 1 mm for approximately 30 minutes. The tip was immersed in a vertical orientation in order to ensure that there was no nonspecific binding due to gravity. Between detections, the antibody surface of each cantilever was regenerated by a brief dip in HCl-glycine solution.

The QCM sensor exhibited a resonance frequency shift of 45 Hz for antibody immobilization; PECS exhibited a resonance frequency shift of 450 Hz. Using the QCM resonance frequency value and the Sauerbrey equation, the mass change per unit area due to the antibody immobilization was determined to be 3.5x10⁻⁹ g/mm². In comparison, the detection sensitivity for a piezoelectric cantilever sensor, shown in FIG. 11(a), was determined to be approximately 3x10⁻¹¹ g/Hz. FIG. 11(b) and Table 2 shows the detection curves for the piezoelectric cantilever and demonstrates that the cantilever is only capable of detecting PSA at 4 µg/ml and above.

**TABLE 2**

<table>
<thead>
<tr>
<th>Detector Type</th>
<th>Detection sensitivity</th>
<th>Label-free</th>
<th>Direct, in-situ</th>
<th>Rapid</th>
<th>High-throughput</th>
<th>Multiplexing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEMS</td>
<td>10⁻¹⁵, 10⁻¹⁶ g</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>QCM</td>
<td>10⁻⁹ g</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
<td>no</td>
</tr>
<tr>
<td>Silicon</td>
<td>10⁻¹² g</td>
<td>yes</td>
<td>no</td>
<td>No</td>
<td>No</td>
<td>no</td>
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<tr>
<td>Optical fiber</td>
<td></td>
<td>no</td>
<td>no</td>
<td>No</td>
<td>Yes</td>
<td>yes</td>
</tr>
<tr>
<td>Fluorescence</td>
<td></td>
<td>no</td>
<td>no</td>
<td>No</td>
<td>Yes</td>
<td>yes</td>
</tr>
<tr>
<td>ELISA²⁶</td>
<td>10⁻¹⁰ g</td>
<td>no</td>
<td>no</td>
<td>No</td>
<td>Yes</td>
<td>yes</td>
</tr>
<tr>
<td>SPR³</td>
<td>10⁻¹² g</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
<td>no</td>
</tr>
</tbody>
</table>

By comparison, the sensitivities of various PMN PEMs were determined by subjecting the PEMs to a controlled 70% humidity and 16°C environment in which they were immersed in a flowing PSA solution of various concentrations. FIG. 12 shows that a 340 μm long, 750 μm wide and 8 μm thick PMN—PT/Cu microcantilever, represented in FIG. 12 by square data points, was found to have a sensitivity of 2x10⁻¹³ g/Hz; a 900 μm long, 750 μm wide and 8 μm thick PMN—PT/Cu microcantilever, represented by triangle data points in FIG. 12, was found to have a sensitivity 3x10⁻¹³ g/Hz; and an 800 μm long, 750 μm wide and 22 μm thick microcantilever tip is then rinsed with distilled water and activated by immersing in a solution containing 5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 5 mg/ml N-hydroxysuccinimide (NHS) and distilled water for 30 min. 1 µl of a 0.1 mg/ml solution containing anti-PSA in PBS at a pH of 7.4 was then coated on the cantilever surface for 30 min.

**Example 3**

There exist a number of means for immobilizing various receptors on various different electrode surfaces. The immobilization methods may differ depending upon the receptor to be bound on the electrode surface. For example, BA antigens may be immobilized on a lead surface by cleaning a platinum electrode with a > 14 diluted piranha solution for 2 min, soaking the PEMs in a 2 mM 3-mercaptoproprionic acid (MPA) for 2 hr to form a MPA monolayer on the electrode surface, and activating the carboxyl group of the MPA using a solution of 2 mM N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM N-hydroxysuccinimide (NHS) in water to allow covalent bonding of primary amines on the anti-BA-spore antibody to the MPA monolayer.

A method for PSA receptor immobilization on a lead surface includes the steps of immersing the microcantilever in a solution containing 1 part 30% H₂O₂, concentrated H₂SO₄ and 29 parts water for 2 min, rinsing with distilled water and immersing in a 1 mM MPA aqueous solution overnight.
solution of 5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 5 mg/ml N-hydroxysulfosuccinimide (NHS) and distilled water for 30 min and partially immersing the microcantilever in the scFv solution. Alternatively, it may be possible to utilize longer mercaptoundecanoic acid (MUA) in order to optimize the efficiency.

scFv receptors may also be immobilized on a gold surface by using Cu²⁺ ions to bind to His6 tag of scFv. In this method, the gold coated microcantilever tip is first cleaned with Piranha solution for 10 minutes and rinsed with distilled water. The gold surface is then treated with MPTS, MPA (mercaptotriopionic acid), or MUA (mercaptoundecanoic acid) to form a monolayer on the gold-coated sensor surface. Preferably, the gold surface is treated by immersion in a solution of 1 mM 11 Mercaptoundecanoic Acid (MUA) and ethanol for 3 hours and rinsed with distilled water. The cantilever is then immersed in a 2 mM CuClO₄ aqueous solution for 10 min to adsorb Cu²⁺ on the MUA self-assembled monolayer and form a MPA-Cu composite monolayer. A monolayer of Cu ions will bind to the His6 tag at the C-terminal of scFv. The cantilever may then be partially immersed in the scFv solution for in-situ monitoring the binding of the scFv to the Cu²⁺ ions on the cantilever tip surface.

Another immobilization method of scFv to a SiO₂ surface entails cleaning an electrode surface using strong acid, reacting the surface with Glycidoxypropyl trimethoxysilane (GOPTS) to generate epoxy groups. The surface is then reacted with amine groups of lysine in scFv. Alternatively, it may be possible to utilize other bifunctional linkers such as alkoxysilane-PEG-carboxyl on the SiO₂ surface. The surface is then treated with Cu(ClO₄)₂·6H₂O or CuCl₂ to create Cu²⁺ ions on the surface that will bind the His6 tag or cysteine tag at the C-terminal of the scFv. The length of the PEG may vary to obtain optimal antigen-receptor binding.

Example 4

The PEMs of the present invention may also be utilized for simultaneous detection of multiple antigens using a piezoelectric cantilever array. In an experiment, three piezoelectric cantilever sensors were utilized for simultaneous detection of three proteins. One sensor was coated with antibodies specific to PSA, a second sensor was coated with scFv specific to HER2 and a third was coated with antibodies specific to gp120. FIGS. 13(a), 13(b) and 13(c) show the resonance frequency shifts of three PEMs over time in various solutions, one coated with scFv of HER2, one coated with an antibody to PSA and one coated with antibody gp120. FIG. 13(c) shows the detection results when the scFv is placed in a HER2 solution of 23 µg/ml. FIG. 13(b) shows the detection results when the scFv is placed in a PSA solution of 18 µg/ml, and FIG. 13(c) shows the detection results when the scFv is placed in a gp120 solution of 100 µg/ml. FIG. 13(a) shows that only the scFv coated with HER2 responded to the HER2 solution. Similarly, only the scFv coated with antibodies to PSA responded to the PSA solution of FIG. 13(b), and only the scFv coated with antibodies to gp120 responded to the gp120 solution.

The experiment demonstrated that only sensors coated with antibodies specific to the antigen solution reacted in each of the three solutions. The experiment proves that a cantilever array can perform in-situ, rapid, simultaneous detection of multiple antigens. A portable device may therefore be used to carry multiple sensors for multiple antigen detection in a solution.

Example 5

A number of methods and electrically insulating materials may be used to insulate the electrodes of a PEMs. Parylene and MTMS, in particular, were separately coated on PZT PEMs and evaluated for dampening impedance.

FIG. 14(a) shows the impedance resonance spectrum of a parylene insulated PMN—PT/Cu PEMS completely immersed in a static and 1 ml/min flow solution of PBS. The PEMS is insulated with a 1.5 µm thick parylene coating and has a mass detection sensitivity of about 4x10⁻¹⁵ g/Hz. The background resonance frequency shift was no more than 30 (60) Hz in 30 min in static PBS and in flowing PBS at 1 ml/min. It is expected that at a typical detection flow rate of 0.2-0.5 ml/min, the background resonance frequency shift should be no more than 40 Hz in 30 min. Note that even at 1 ml/min, the resonance peak still retained a Q value well over 35.

FIG. 14 (b) compares the peak position shift with time in static PBS and in a 1 ml/min flow. As can be seen the peak positions were fairly stable with no more than 30 Hz shift over 30 minutes in static PBS and no more than a 60 Hz shift over 30 minutes at a flow rate of 1 ml/min in PBS. At the typical flow rate 0.2-0.5 ml/min, the background resonance frequency shift is expected to be no more than 40 Hz.

FIG. 15(a) shows the resonance spectrum of a PZT PEMS insulated with a monolayer of MTMS. The PEMS was placed in a flow cell and completely immersed in a 0.5 ml/min flow solution of PBS. It was found to have a mass detection sensitivity of about 5x10⁻¹¹ g/Hz. FIG. 15(b) shows the background resonance frequency shift as a function of time in flowing PBS at 0.5 ml/min. The background resonance frequency shift was no more than 30 Hz over 30 minutes. The graphs show that the electrical impedance spectrum was stable with strong resonance peak intensity and indicate that the MTMS monolayer is a good electrical insulation layer.

Example 6

A PMN—PT piezoelectric layer or film may be fabricated using a precursor-suspension method. Submicron crystalline PMN powder was prepared by dispersing Mg(OH)₂-coated Nb₂O₅ particles in a lead acetate/ethylene glycol solution followed by calcination at about 800°C. The crystalline PMN powder was subsequently suspended in a lead precursor solution containing lead acetate and titanium isopropoxide in ethylene glycol to form a PMN—PT precursor powder, which can be sintered at a temperature as low as about 900°C.

The PMN—PT films were then polarized in order to obtain high piezoelectric coefficients. Before polarization, the orientations of domains were random with no net polarization. After polarization, many domains were aligned in the direction of the applied electric field resulting in a finite polarization. Although polycrystalline materials are not easily aligned in comparison to single crystal materials, PMN—PT films were geometrically structured so as to facilitate polarization alignment within the plane of the film.

The resulting freestanding PMN—PT films were then electroplated on one side with a sputtered 30 nm thick nickel/ platinum (Ti/Pt) layer. A copper layer of appropriate thickness was then electroplated on the Pt surface to create a non-piezoelectric layer, followed by the deposition of the Ti/Pt electrode on the other face of the film. A PMN—Pt/Cu bi-layer was then embedded in wax and cut or chemically etched to form a cantilever shape with a wire saw.

A particularly advantageous aspect of this method is the ability to construct a microcantilever from one piece of PMN—PT film without having to separately attach the non-piezoelectric and piezoelectric layers, facilitating the manufacturing process. Because the cantilevers, thus fabricated, had a clean geometry and clean interfaces between the
PMN—PT layer and the electrodes, cantilever sensitivity was heightened, as demonstrated by Q values as high as 300. The resulting suspensions have previously been used to formulate PMN—PT freestanding films of about 8-75 \( \mu \)m thick upon sintering at 1000°C. Typically, the freestanding PMN—PT films have femtogram sensitivity of at least \( 2 \times 10^{-14} \) g/Hz, dielectric constants greater than 1000, saturated polarization of about 30 \( \mu \)C/cm², remnant polarization of 25 \( \mu \)C/cm² and a Q value as high as 300. Therefore PMN—PT microcantilevers are capable of generating higher-mode resonance peaks resulting in enhanced sensitivity detection.

Example 7

A PZT/SiO₂ piezoelectric layer of film may be formed on silicon wafers and attached to a substrate, such as glass, to form an array. FIG. 3 depicts a preferred micro-fabrication procedure for constructing PZT/SiO₂ piezoelectric microcantilever sensors. A low-stress SiO₂ substrate, 2-\( \mu \)m in thickness, was first deposited on front and back sides of a (100)-oriented silicon wafer by low energy sputtering of \( \text{Ar} \) and \( \text{O}_2 \). A thin film of TiO₂ on the wafer's back side was then deposited by reactive sputtering of Ti followed by the deposition of a 1500 \( \AA \) thick Pt electrode. Reactive sputtering of Ti was carried out under 15 mTorr of 80% oxygen and 20% helium. To prepare for deposition of the platinum electrode, a stage or platform was heated to 650°C at a rate of 5°C/min. The RF power was adjusted to the expected deposition rate. After deposition, the stage was cooled to room temperature at a rate of 5°C/min to avoid resistivity stresses. Sputtering of the platinum bottom electrode began when the stage reached room temperature. The resulting platinum bottom electrode was preferably about 1500 \( \AA \) in thickness.

A 1.5-\( \mu \)m thick PtZT layer was then deposited on the Pt/TiO₂/SiO₂/Si substrate using a sol gel method with repeated spin coating and heat treatment steps. According to the sol gel method, titanium isopropanoxide and lead acetate were dissolved in ethylene glycol and zirconium-n-propoxide in 2-propanol. The dissolved components were mixed to obtain a PZT precursor solution. The precursor solution also contained a 50% excess of lead to compensate for lead loss during repeated heat treatment. The PZT thin films were then deposited by repeated spin coating on a Pt/TiO₂/SiO₂/Si substrate followed by pyrolysis at 350°C for 12 min and sintering at 650°C for 2 hours after every 5 depositions. The Pt/TiO₂/SiO₂ substrate was then sintered at 900°C for 1 hour to ensure the Pt/TiO₂/SiO₂/Si substrate was ready for the PZT deposition.

After forming the PZT layer, a first electrode, Pt/Ti or Au/Cr, was deposited by E-gun evaporation in high vacuum (2x10⁻¹⁰ torr or lower) to avoid oxidation of metal, particularly oxidation of the Cr layer. E-gun evaporation was used because it is gentler than sputtering or thermal evaporation and avoids damaging the PZT layer. The thickness of the resultant Ti or Cr bonding layer was approximately 5-40 nm and the resultant Pt or Au electrode layer was approximately 100-200 nm thick. A nickel layer was deposited on the top electrode to form a hard mask for protecting the top electrode and the underlying PZT during dry etching. The Ni and top electrode were patterned using an over-hang lift-off process. LOR10B and SPR3012 photoresists are recommended for executing the over-hang lift-off process, and the thickness of the undercoat photoresist (LOR) was about 0.8 to 1.2 \( \mu \)m, approximately 1.5 times larger than the metal layer. The selectivity or etching rate ratio of PZT to Ni was nearly 5:1. The thickness of the resultant Ni layer was about 0.3-0.5 \( \mu \)m, depending on the PZT thickness.

The exposed PZT and TiO₂/Pt bottom electrode was patterned by a chlorine-based inductively coupled plasma (ICP) dry etching process using a chlorine etching gas to expose the SiO₂ and 150 secm flow rate. The pressure of the etching chamber was set to below 10 mTorr. The RF power was adjusted to the etching rate. Since the PZT is a ceramic material and the nickel etching mask is stable at high temperatures, a high RF power of about 400 W is recommended for PZT dry etching. The selectivity ratios of the PZT dry etching process were 5:1 for Ni and 8:1 for Pt. The etching mask for the bottom electrode was a thick photoresist such as one in the SPR220 series. Because the etching mask was a photoresist, a low RF power was recommended for this etching process. Silver glue was used to fill spaces between the sample and the stage to help dissipate heat during etching. For every 5 min of etching, a 20-min cooling period was required. By keeping the etching temperature below 120°C, the PR was easily removed with acetone after etching.

The silicon dioxide on the second side was patterned by photolithography, and the exposed silicon dioxide was etched by CF₄/CHF₃ based reactive ion etching (RIE). After the silicon-dioxide etching was finished, the whole first side was sealed by black wax, and the sample was placed in a 45% potassium hydroxide (KOH) solution. The temperature of the solution was set to 55-60°C, and the KOH was used to etch the exposed silicon with the remaining silicon dioxide as an etching mask.

After etching, the black wax was dissolved using trichloroethylene (TCE) and the front side was cleaned. The exposed silicon dioxide was removed on the front side by a CF₄/CHF₃-based reactive ion etching (RIE). The first electrode and second electrode work together because the mask and the selectivity ratios are very high. If desired, the etching duration may be increased to ensure that all the silicon dioxide is removed and the cantilevers are released. After rinsing with acetone, 2-propanol and deionized water, free-standing PZT/SiO₂ piezoelectric microcantilever sensors were obtained.

Example 8

Mercaptopropylsilane (MPTMS) was investigated for its ability to function both as an electrical insulator and as receptor immobilizer. The results established that MPTMS is an effective insulator and immobilizer enabling the detection of the HER2 antibody both in solution and in the presence of BSA.

To prepare the MPTMS coated PEMs, a PMN—PT/tin PEMs was first soaked in a diluted (1:100 in water) piranha solution, containing two parts 98% sulfuric acid and one part of 30% hydrogen peroxide, at 20°C. For 1 min to clean the tin and gold surfaces. Next, the PEMs was submerged in a beaker containing a 40 mM solution of MPTMS in ethanol, and the beaker was covered with paraffin film to prevent ethanol evaporation. After 4 hours, the PEMs was rinsed with DI water and submerged in a 0.01 M solution of NaOH overnight for cross-linking. The PEMs was then soaked in DI water for 1 hour and dried overnight in a vacuum oven at 762 mm Hg to ensure all the water was removed. The PEMs was then sub-
merged in a 1 volume percent solution of MPTS in ethanol titrated to a pH 4.5 with acetic acid. The solution was covered with parafilm to prevent ethanol evaporation, and the PEMs was allowed to soak for 8 hours. The PEMs was again soaked in DI water for 1 hour and dried overnight in a vacuum oven at 762 mm Hg.

To immobilize the scFv receptors on the PEMs surface, sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SUfo-SMCC) was first mixed with scFv to form a peptide bond between a primary amine of the scFv and the NHS ester of the SUfo-SMCC as depicted in FIG. 16(a). To effectively bind SMCC to scFv, 5 mM SMCC was mixed with 400 nM scFv for 2 hrs. The unreacted SMCC was then removed by repeated microcentrifugation with a 10 kD filter a total of 4 times. The MPTS-coated PEMs was then soaked in the scFv-bound bound SMCC solution.

FIG. 16(b) illustrates the reaction between the sulfhydryl of the MPTS on the sensor surface with the maleimide of the scFv-bound SMCC to immobilize the scFv. The immobilized scFv has random orientation. After immobilization, the PEMs was placed in a mild quenching solution of DI water for 1 hr to quench the unreacted NHS functionalities on the SMCC. Since any primary amine of the scFv could react with the NHS ester of the SMCC the resultant immobilized scFv would be randomly oriented as schematically shown in FIG. 16(b). With the cysteine tug engineered at the base of the scFv, it is now possible to covalently bind the scFv to the sensor surface and at the same time orient the scFv for optical antigen-scFv binding.

For HER2 detection, the scFv-immobilized PMN—PT/tin PEMs was immersed in a home-built flow cell with a peristaltic pump. First PBS was allowed to flow across the conjugated surface for 10 minutes in order to obtain a background reading. Next, a blocking solution of 10 mg/ml of BSA in PBS was allowed to flow over the surface of the cantilever until no significant deviations are observed in the cantilevers resonant frequency. The cantilever was then gently rinsed with a solution of TWEEN-20 followed by PBS. PBS was then allowed to flow across the surface of the cantilever for 10 minutes to ensure that there was no background drift. The flow cell contained 3 ml of HER2 solution. The detection was carried out with two faces of the PEMs tangential to the flow at a flow rate of 0.5 ml/min. After each detection, the HER2 was released from the PEMs surface by flushing with a glycine/TrCl solution having a pH of about 2.5 or a supersaturated solution of NaCl. Following the release, the PEMs was then exposed to a suspension of a different concentration for another detection run.

FIGS. 17(a)-17(c) show the in-situ results of scFv immobilization, the subsequent BSA blocking and detection of 100 ng/ml of HER2 along with the background environment of BSA, using the MPTS-insulated PMN—PT/Sn PEMs. FIG. 17(b) shows that the MPTS-coating was effective as an electrical layer as the resonance peaks maintained the same high Q value throughout the 2 hour detection period. In addition, FIGS. 17(a) and 17(c) illustrate that HER2 can be detected in the background environment of BSA. After BSA blocking, the resonance frequency shift for detecting HER2 alone, FIG. 17(a), and in the presence of BSA, (FIG. 17(c)), was roughly the same, indicating that the BSA blocking procedure effectively saturated the nonspecific binding sites of BSA and had no observable effect to the PEMs resonance frequency shift. FIG. 18 shows the resonance frequency shift versus time in HER2 detection at 5, 10, and 50 ng/ml in a background of 1 mg/ml BSA using the MPTS-insulated PMN—PT/Sn PEMS. As can be seen, the detection yielded ~50 Hz, ~150 Hz, and ~700 Hz after 50 min at 5, 10, and 50 ng/ml, respectively, indicating that the present PMN—PT/Sn PEMs is capable of detecting HER2 in a background of BSA whose concentration was one million fold higher than that of HER2.

**Example 9**

The PEMs is capable of in situ detection applications. A one-sided PEMs was able to detect the presence of HER2 in the presence of Bovine Serum Albumin (BSA).

The tin surface of a PMN—PT/Sn PEMs was insulated using MTMS. The scFv receptors were immobilized only on the platinum side of the electrode by first coating the platinum surface with an activated MPA self assembly monolayer to bind to a primary amine of the scFv.

A one sided PMN—PT/tin PEMs was found capable of detecting of HER2 at a concentration of 1 ng/ml in the presence of 1 mg/ml of BSA in a 30 ml solution flowing at a rate of 1 ml/min. FIG. 19 shows a resonance increase in the presence of HER2, indicating that the PEMs was capable of detecting 1 ng/ml of HER2 in 1 mg/ml of BSA.

The BSA did not adhere to the MPA surface to the same degree as compared to the MPTS—SMCC surface of FIGS. 17(a) and 17(c). In the presence of 1 mg/ml of BSA, the resonance frequency of the PMN—PT/tin PEMs was stable as shown at t=150 of FIG. 19. In comparison, in the presence of 1 ng/ml of HER2 and 1 mg/ml of BSA, the resonance frequency of the PEMs increased by about 200 Hz during a 160<sub>t</sub><sub>c</sub>240 interval. Upon the removal of the HER2, the resonance frequency stabilized, suggesting that the one-sided PMN—PT/tin was able to detect the presence of HER2 at 1 ng/ml in the presence of 1 mg/ml of BSA. Note that resonance frequency increase upon binding of target antigens on the sensor surface is common with one-sided detection due to the stress imbalance from the one-sided binding.

FIGS. 17-19 clearly demonstrate that PMN—PT/tin PEMs were able to detect HER2 in situ at a concentration as low as 1 ng/ml in the presence of a 1 mg/ml BSA background, which is lower than the normal 15 mg/ml HER2 concentration of a typical healthy woman. Therefore, these results suggest that PEMs would be able to detect breast cancer markers in serum with useful levels of sensitivity and specificity.

**Example 10**

A 900 µm long and 500 µm wide PMN—PT/Cu PEMs having an 8 µm thick PMN—PT layer and a 3 µm thick copper layer was used to detect the presence of HER2 in diluted fetal bovine serum (FBS).

The PEMs was prepared using mercaptopropyltrimethoxysilane (MPS) as an insulating layer and covalent receptor conjugation. Biotin was covalently bound to the sulfhydryl of the MPS using maleimide-PEO<sub>2</sub>-biotin (Pierce). Avidin was subsequently attached to the biotin by soaking the surface of the cantilever in a 1 mg/ml solution of neutravidin (Pierce). Sulfosuccinimidyldi-6-[biotinamido]-6-hexanamido hexaoate (Pierce) was used to attach biotin to the primary amine in a scFv. The avidin coated PEMs was then submerged in the solution containing the biotinylated scFv for 60 minutes at 4°C. The cantilever was subsequently soaked in a 3% BSA solution for 2 hours to facilitate blocking of unreacted areas. The PEMs was then rinsed with a 1% BSA solution and a 1% Tween® 20 solution.

For HER2 detection, the scFv-immobilized PEMs was then immersed in a flow cell containing about 6 ml of liquid with a peristaltic pump. After conjugation, the PEMs was exposed to a solution containing diluted 1:40 bovine serum
and HER2 in one of the following concentrations: about 110 pg/ml, 50 pg/ml, 30 pg/ml, and 10 pg/ml. FIG. 29 shows the resonance frequency shift versus time for the PEMS. As can be seen, the PEMS yielded a resonance frequency shift of about 2800 ± 200, 1000 ± 100, 140 ± 100, and 10 ± 100 Hz at t=50 minutes, for 110, 50, 30 and 10 ng/ml HER2 concentrations, respectively. The results indicated that the PEMS were able to detect breast cancer markers in diluted serum with a concentration limit of 30 pg/ml.

Example 12

An array of three millimeter long PZT/glass PEMS were used to assess the binding ability of a receptor for its ligand. The performance of 3 receptors, B1 scFv, H3 scFv, and Herceptin, was compared using an array of three identical PZT/glass PEMS insulated with MPS. A heterobifunctional cross linking agent, sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate, was used to covalently attach the receptor to the MPS surface. After receptor immobilization the cantilevers were blocked using a 3% BSA solution for 1 hour, and then rinsed with a 1% BSA solution and a 1% Tween® 20 solution.

The three PEMS were all completely submerged in the same 6 ml flow cell. A 1-40 fetal bovine serum containing a known spiked amount of HER2 ECD was introduced into the flow cell. The sample was flowed through the system at a rate of 0.7 ml/minute. FIG. 21 shows the resultant PEMS detection of HER2 in solutions containing diluted 1:40 fetal bovine serum and HER2 in concentrations of 6 ng/ml, 60 ng/ml or 600 ng/ml. In Table 3, the resonance frequency shift is given at t=60 minutes. According to FIG. 21 and Table 3, three identical PEMS showed that Herceptin consistently had lower frequency shifts, suggesting that Herceptin can be detected using PEMS at a lower concentration limit, as compared to B1 scFv and H3 scFv.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>H3</th>
<th>B1</th>
<th>Herceptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ng/ml</td>
<td>55</td>
<td>460</td>
<td>850</td>
</tr>
<tr>
<td>60 ng/ml</td>
<td>150</td>
<td>840</td>
<td>1500</td>
</tr>
<tr>
<td>600 ng/ml</td>
<td>780</td>
<td>1300</td>
<td>3200</td>
</tr>
</tbody>
</table>

Example 13

A 40 µm long PZT/SiO₂ PEMS, as shown in FIG. 23(a), was found to have an improved sensitivity of about 10⁻¹⁶ g/Hz with resonance peak Q values as high as 300 in a humidity controlled environment. FIG. 23(b) shows the resonance frequency spectra of the PEMS corresponding to varying humidity. The resonance frequency was found to decrease with an increasing relative humidity due to the adsorption of water molecules on the sensor surface. Therefore it is possible to increase sensitivity with very small PEMS.

The resultant sensitivity of 6x10⁻¹⁶ g/Hz demonstrates the feasibility of further reducing PEMS size while increasing sensitivity when compared to the millimeter sized commercial PZT PEMS having a sensitivity of about 10⁻¹¹ g/Hz and 300-800 µm long PMN—PT PEMS having a sensitivity of about 10⁻¹³ to 10⁻¹⁴ g/Hz.

Having described the preferred embodiments of the invention which are intended to be illustrative and not limiting, it is noted that modifications and variations can be made by persons skilled in the art in light of the above teachings. It is therefore to be understood that changes may be made in the particular embodiments of the invention disclosed which are within the scope and spirit of the invention as outlined by the appended claims. Having thus described the invention with the details and particularity required by the patent laws, the intended scope of protection is set forth in the appended claims.

The invention claimed is:

1. A method for using a piezoelectric microcantilever sensor comprising the steps of:
   - actuating a microcantilever sensor having a Q value greater than 20, comprising a piezoelectric layer, a non-piezoelectric layer, and a receptor, wherein said piezoelectric layer has a thickness of about 1 µm to about 127 µm, a dielectric constant of at least 1600, and a piezoelectric coefficient ~d₃₃ of at least about 250 pm/V;
   - binding a molecule or compound to said receptor, detecting a force exerted on said piezoelectric layer by said bound molecule or compound, and determining one or more of a presence of said molecule or compound and a concentration of said molecule or compound from said detected force.
2. The method of claim 1, wherein said receptor is selected from the group consisting of DNA, proteins, enzymes, cells, viruses, parasites, antigens and pathogens.
3. The method of claim 1, wherein said molecule or compound is selected from the group consisting of bacteriostatic agents, cancer agents, bacterial disease agents and viral disease agents.
4. The method of claim 1, further comprising the step of detecting a resonance frequency shift.
5. The method of claim 1, wherein the microcantilever sensor further comprises an electrical insulation layer which insulates conducting elements of said sensor.
6. The method of claim 5, wherein the electrical insulation layer comprises a material selected from the group consisting of poly-para-xylene, methylmethoxysilane, 3-mercaptopropyl trimethoxysilane, Al₂O₃, SiO₂ and functionalized hydrophobic silanes and mixtures thereof.
7. The method of claim 1, wherein the receptor is bound to a conducting element by an immobilization layer.

8. The method of claim 1, wherein a length of said piezoelectric layer is less than or greater than a length of said non-piezoelectric layer.

9. The method of claim 1, wherein said piezoelectric layer if fabricated from a freestanding film.

10. The method of claim 1, wherein said piezoelectric layer has a thickness of less than 2 µm.

11. The method of claim 1, wherein said piezoelectric layer has a dielectric constant of at least 1900 and a thickness less than 4 µm.

12. The method of claim 1, wherein said piezoelectric layer has a dielectric constant of at least 1900 and a thickness less than 2 µm.

13. The method of claim 1, wherein said piezoelectric layer has a thickness less than 8 µm.

14. The method of claim 1, wherein said piezoelectric layer comprises a material selected from the group consisting of lead magnesium niobate-lead titanate, lead-zirconate-titanate, and doped sodium potassium niobate-lithium niobate.

15. The method of claim 1, wherein said piezoelectric layer comprises a lead-free piezoelectric material.

16. The method of claim 1, wherein said piezoelectric microcantilever has a Q value greater than 120.

17. The method of claim 1, wherein the non-piezoelectric layer comprises a material selected from the group consisting of a ceramic material, a polymeric material, a metallic material and a combinations thereof.

18. The method of claim 1, wherein said non-piezoelectric layer comprises a material selected from the group consisting of silicon dioxide, silicon nitride, tin and copper.

19. The method of claim 1, wherein said non-piezoelectric layer comprises multiple layers of different materials.

20. The method of claim 1, wherein the step of actuating comprises applying a voltage across said piezoelectric layer.